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Virulence potential of *Staphylococcus aureus* isolates from Buruli ulcer patients

Nana Ama Amissah[a,b,*], Monika A. Chlebowicz[c], Anthony Ablordey[b], Caitlin S. Tetteh[b], Isaac Prah[b], Tjip S. van der werf[a], Alex W. Friedrich[c], Jan Maarten van Dijl[c], Ymkje Stienstra[a,1], John W. Rossen[c,1]

[a] Department of Internal Medicine/Infectious Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[b] Department of Bacteriology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana
[c] Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

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ABSTRACT

Buruli ulcer (BU) is a necrotizing infection of the skin and subcutaneous tissue caused by *Mycobacterium ulcerans*. BU wounds may also be colonized with other microorganisms including *Staphylococcus aureus*. This study aimed to characterize the virulence factors of *S. aureus* isolated from BU patients. Previously sequenced genomes of 21 *S. aureus* isolates from BU patients were screened for the presence of virulence genes. The results show that all *S. aureus* isolates harbored on their core genomes genes for known virulence factors like α-hemolysin, and the α- and β-phenol soluble modulins. Besides the core genome virulence genes, mobile genetic elements (MGEs), i.e. prophages, genomic islands, pathogenicity islands and a Staphylococcal cassette chromosome (SCC) were found to carry different combinations of virulence factors, among them genes that are known to encode factors that promote immune evasion, superantigens and Panton-Valentine Leucocidin. The present observations imply that the *S. aureus* isolates from BU patients harbor a diverse repertoire of virulence genes that may enhance bacterial survival and persistence in the wound environment and potentially contribute to delayed wound healing.

1. Introduction

Staphylococcus aureus is one of the most common bacteria residing in chronic wounds, including the Buruli ulcers (BU) caused by *Mycobacterium ulcerans* (Amissah et al., 2015b; Barogui et al., 2013). The presence of *S. aureus* is a potential risk for wound infections, especially if they produce virulence factors that outweigh the hosts' ability to resist them. In fact, *S. aureus* is notorious for producing a range of virulence factors that are involved in the persistence of colonization, infection, tissue damage and delayed wound healing (Bessa et al., 2015).

The major known virulence factors of *S. aureus* include cytolytic toxins such as the hemolysins, α-toxin, various leucocidins (e.g. Panton-Valentine Leucocidin [PVL] and exofoliative toxins [ETA and ETB]), and phenol-soluble modulins [PSMs]), as well as superantigens such as the toxic shock syndrome toxin-1 (TSST-1) (Prévost et al., 2001; Wang et al., 2007). Production of particular virulence factors by *S. aureus* can sometimes be related to specific diseases. This is exemplified by the toxic shock syndrome caused by TSST-1-positive isolates, or the staphylococcal scalded skin syndrome caused by ETA- or ETB-positive isolates (Todd et al., 1978). However, in general the severity of *S. aureus* infection seems to be related to the range and amounts of different toxins that are simultaneously produced (Otto, 2012). In addition, other virulence factors enhance the capacity of *S. aureus* to survive in the human host. These include immune evasion factors, such as staphylokinase (Sak), the staphylococcal inhibitor of complement (SCIN), and the chemotaxis inhibitory protein (CHIPS). Most often these virulence factors are encoded on mobile genetic elements (MGEs), such as prophages, plasmids, genomic islands, staphylococcal cassette chromosome (SCC) elements, and *S. aureus* pathogenicity islands (SaPIs) (Novick, 2003). Additionally, the genome of *S. aureus* contains specific genes with a function in pathogenesis and host adaptation.

Abbreviations: BU, Buruli ulcer; MGEs, mobile genetic elements; SCC, Staphylococcal cassette chromosome; PVL, Panton-Valentine Leucocidin; ETA and ETB, exofoliative toxins; PSMs, phenol-soluble modulins; TSST-1, toxic shock syndrome toxin-1; Sak, staphylokinase; SCIN, staphylococcal inhibitor of complement; CHIPS, chemotaxis inhibitory protein; SaPIs, *Staphylococcus aureus* pathogenicity islands; agr, accessory gene regulator; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin susceptible *S. aureus*; hla, hlb and hld, hemolysins; ORFs, open reading frames; egc, enterotoxin gene cluster; νSaα and νSaβ, genomic islands; IEC, immune evasion gene cluster

* Corresponding author at: University of Ghana, Noguchi Memorial Institute for Medical Research, P. O. Box LG 581, Accra, Ghana.
E-mail address: namissah@noguchi.ug.edu.gh (N.A. Amissah).
[1] These authors contributed equally to this work.

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Many of these are controlled by the accessory gene regulator (*agr*) system that has a major impact on the virulence of *S. aureus* (Novick et al., 1993).

PVL is one of the prophage-encoded virulence factors implicated in *S. aureus* necrotic skin lesions (Novick, 2003; Novick et al., 1993). The PVL genes have been detected in 53–62% of *S. aureus* isolates from skin and soft tissue infections (Kilic et al., 2015; Nurjadi et al., 2015; Pardos de la Gandara et al., 2015). In a recent study, we observed that 79% of the *S. aureus* isolates from wounds of BU patients treated with streptomycin and rifampicin were PVL-positive (Amissah et al., 2015b). Since *M. ulcerans* is effectively killed upon this antibiotic therapy, we hypothesized that the wound-resident *S. aureus* may still affect the soft tissue, thereby causing a delay in wound healing. As PVL is one of many known *S. aureus* virulence factors, the present study aimed to reveal all virulence genes present in *S. aureus* isolates from BU patients. To this end, the genomes of 21 *S. aureus* isolates from the anterior nares and wounds of BU patients were sequenced and analyzed for the presence of known virulence genes.

2. Materials and methods

2.1. Ethical statement

The study was approved by the ethics committee of the Noguchi Memorial Institute for Medical Research (FEDERAL WIDE ASSURANCE FWA 00001824), and was carried out in accordance with the approval guidelines. All samples were collected upon written informed consent from adult subjects, or a parent or guardian of any child participant on behalf of the respective child below 18 years. Specifically, samples were collected from the anterior nares and wounds of eleven BU patients who received treatment at the Pakro Health Center in the Eastern region of Ghana.

2.2. Bacterial isolates

The 21 *S. aureus* isolates used in this study are listed in Table 1. Their isolation and initial characterization was described (Amissah et al., 2015a). Specifically, four isolates were obtained from the anterior nares and 17 from the wounds of eleven BU patients. These included six methicillin resistant *S. aureus* (MRSA) and 15 methicillin susceptible *S. aureus* (MSSA) isolates (Table 1).

2.3. Hemolytic activity of S. aureus isolates from BU patients

S. aureus isolates were grown overnight in 3 ml of Tryptic Soy Broth (TSB) at 37 °C. A 10 µl loopful of the *S. aureus* RN4220 control strain was streaked vertically at the center of freshly prepared 5% sheep blood agar (BA) plates. Next, the 21 investigated *S. aureus* isolates from BU patients were streaked perpendicularly on both sides of the RN4220 streak. Plates were incubated overnight at 37 °C after which the possible synergistic hemolytic activity was assessed by visual inspection of clearance zones due to the lysis of red blood cells.

2.4. Whole genome sequencing, sequence assembly and data analyses

Genomic DNA for whole genome sequencing (WGS) was obtained from *S. aureus* isolates as previously described (Amissah et al., 2015a). DNA libraries were prepared using the Nextera XT v2 kit (Illumina, San Diego, CA, USA) according to the manufacturers' instructions and then run on a Miseq (Illumina), which resulted in paired-end reads of ~250-bp. *De novo* sequence assembly was performed using the CLC Genomics Workbench v7.0.4 package (CLC bio A/S, Aarhus, Denmark) after quality trimming (Qs > 28) with optimal word sizes based on the maximum N50 value. The sequence reads were submitted to the National Center for Biotechnology Information GenBank and are available under the BioProject PRJNA283747, SRP (raw reads) study accession: SRP061319 and accession numbers: LGAE00000000, LFTW00000000, LFTV00000000, LFTU00000000, LFTT00000000, LFOH00000000, LFOG00000000, LFNS00000000, LFNR00000000, LFNQ00000000, LFNP00000000, LFNO00000000, LFNN00000000, LFNM00000000, LFNL00000000, LFNK00000000, LFNJ00000000, LFNI00000000, LFNH00000000, LFMH00000000, LFMG00000000.

2.5. Screening for virulence genes and mobile genetic elements

De novo assembled genomes of sequenced *S. aureus* isolates were queried against specific features of previously sequenced isolates, or compared to the complete *S. aureus* reference genome of MRSA252 with associated annotated genes (NCBI number: BX571856.1) using blastN

Table 1
Detection of genes for hemolysins and phenol soluble modulins.

Patient no.	ST	Strain[a]	Source of sample	*agr* type	*hla*[b,c]	*hlb*[b,d]	*hld*[b]	*Psm*-α[b]	*Psm*-β[b]
2	88	BU_G0201_t8*	Wound	III	+	a	+	+	+
7	88	BU_G0701_t5*	Wound	III	+	a	+	+	+
2	88	BU_G0202_t2*	Wound	III	+	a	+	+	+
19	88	BU_G1905_t3*	Wound	III	+	a	+	+	+
13	88	BU_W13_t1*	Wound	III	+	a	+	+	+
22	5	BU_W22_t4	Wound	II	+	a	+	+	+
7	5	BU_W7A_t11*	Wound	II	+	a	+	+	+
17	15	BU_N17Y_t2	Nose	II	+	b	+	+	+
3	15	BU_N3_t2	Nose	II	+	b	+	+	+
6	1	BU_W6_t1	Wound	III	m	a	+	+	+
12	121	BU_G1201_t13	Wound	IV	+	a	+	+	+
12	121	BU_G1201_t8	Wound	IV	+	a	+	+	+
26	121	BU_G2601A_t9	Wound	IV	+	a	+	+	+
22	3019	BU_N22_t6	Nose	IV	+	a	+	+	+
12	508	BU_W12_t13	Wound	IV	+	a	+	+	+
3	152	BU_G0301_t8	Wound	IV	+	+	+	+	+
10	152	BU_G1074_t4	Wound	IV	+	+	+	+	+
11	152	BU_G1101_t2	Wound	IV	+	+	+	+	+
10	152	BU_G1001_t8	Wound	IV	+	+	+	+	+
17	152	BU_N17W_t2	Nose	IV	+	+	+	+	+
7	152	BU_G0706B_t8	Wound	IV	+	+	+	+	+

[a] MRSA isolates are indicated by an asterisk.
[b] Detection of hemolysins and phenol soluble modulin genes is indicated by +.
[c] A frame shift mutation in the *hla* gene is marked by m.
[d] Insertion of a prophage and IEC genes in the *hlb* gene are marked a and b.

in the WebACT comparison tool (http://www.webact.org/WebACT/prebuilt#). Subsequent detailed analyses were performed with the Artemis Comparison Tool (ACT) software (Carver et al., 2005). Similarity matches were filtered based on their length (100 kb segments) and percentage similarity scores, and only the filtered hits with at least 80% sequence similarity were then displayed by ACT (e-value of 10.00000) and analyzed in detail. Sequence data were queried for the presence of staphylococcal enterotoxin genes (*sea*, *seb*, *sec*1, *sec*3, *sec*4, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep* and *seq*), the toxic shock syndrome toxin-1 gene (*tsst*-1), exfoliative toxin genes (*eta* and *etb*), cytolytic toxin genes, phenol-soluble modulin genes (*psm*-α, *psm*-β and *psm*-γ) and PVL genes (*luk*F-PV and *luk*S-PV). In addition, the presence of genes encoding proteins that have impact on the innate and adaptive immune system was assessed, including genes for the chemotaxis inhibitory protein (*chp*), staphylokinase (*sak*) and staphylococcal complement inhibitor (*scn*). Lastly, the presence of known and potential prophages, genomic islands, SaPIs and SCC*mec* elements was investigated by similarity searches.

3. Results

3.1. Dynamics of S. aureus in wounds of BU patients and wound healing time

As reported previously we determined *S. aureus* diversity over time in the wounds of 19 BU patients (Amissah et al., 2015b). Most of the patients were diagnosed with category II wounds (i.e. lesions between 5 and 15 cm) and time to healing ranged from 2.75 to more than 6 months (Table 2). In most cases, the patients' wounds were colonized by MSSA, but MRSA was detected in the wounds of five patients. The investigated wounds were colonized by *S. aureus* before, during, and after antibiotic treatment (Amissah et al., 2015b). We observed that three patients (patients 2, 10 and 11) were colonized with a single *S. aureus* genotype over time, while two other patients (patients 13 and 19) were found to be positive with *S. aureus* only once during the study period (Amissah et al., 2015b). Two different *S. aureus* genotypes were identified simultaneously at the same time point in the wounds of three patients (patients 6, 12 and 26). Remarkably, the wounds of three patients (patients 3, 22 and 7) were colonized over time with three, four or even six different *S. aureus* genotypes, respectively.

3.2. Detection of hemolysin and phenol soluble modulin genes

To assess the virulence genes of *S. aureus* isolates from the wounds of BU patients, we first queried the core genome for generally well-conserved virulence genes, including those that encode α, β, and δ-hemolysins (*hla*, *hlb* and *hld*) and phenol soluble modulins (*psm*-α and *psm*-β). All investigated isolates harbored the *hla* gene. However, in one isolate with sequence type 1 (ST1), it contained a frame shift mutation caused by a nucleotide deletion (Table 1). The *hlb* gene was found to be intact in all isolates that belonged to ST152, while in most other isolates this gene was split into two parts by insertion of a prophage (φSa3). However, in isolates belonging to ST15, the *hlb* gene was split by an immune evasion gene cluster (IEC). The *hld* and the *psm*-α and *psm*-β genes were present in all isolates (Table 1).

Despite the presence of *hla* encoding α-hemolysin in the genomes of almost all sequenced isolates, α-hemolysin activity was observed only in isolates that belonged to ST152 (Table 2). This may relate to the limited sensitivity of the applied assay for weak α-hemolysin activity. β-hemolysin activity was detected in ten isolates of which four belonged to ST88 and six to ST152 (Table 2). The detection of a β-hemolysin-like activity in the four ST88 isolates was unexpected as they lack an intact *hlb* gene due to phage integration (Table 1). Lastly, 13 isolates displayed δ-hemolysin activity (Table 2). Of note, two isolates belonging to ST121 and ST508, respectively, did not display any hemolytic activity, despite the presence of the *hla* and *hld* genes (Tables 1 and 2).

3.3. Virulence genes located on mobile genetic elements

To gain further insight into the genomic diversity of *S. aureus* isolates from BU patients and the features that may influence host colonization and infection, the genomes of the 21 sequenced isolates were analyzed for the presence of mobile genetic elements that could potentially encode virulence factors (Table 3). Indeed, 20 isolates carried at least one prophage, while in one isolate belonging to ST15 no intact prophage was detected. Different combinations of virulence

Table 2
Investigated *S. aureus* isolates and their hemolytic activity.

Patient no.	ST[a]	Strain	Source of sample	Category of lesion[b]	Time of culture isolation after start of treatment (weeks)	Time to wound healing (months)[c]	Hla[d]	Hlb[d]	Hld[d]
2	88	BU_G0201_t8	Wound	II	16	> 6*		+	+
7	88	BU_G0701_t5	Wound	II	7	6		+	+
2	88	BU_G0202_t2	Wound	II	4	> 6*		+	+
19	88	BU_G1905_t3	Wound	II	2	> 6		+	+
13	88	BU_W13_t1	Wound	II	0	4.5			+
22	5	BU_W22_t4	Wound	III	7	> 6*			+
7	5	BU_W7A_t11	Wound	II	20	6			+
17	15	BU_N17Y_t2	Nose	N/A	N/A	N/A			+
3	15	BU_N3_t2	Nose	N/A	N/A	N/A			+
6	1	BU_W6_t1	Wound	III	0	4.5			+
12	121	BU_G1201_t13	Wound	II	26	> 6*			+
12	121	BU_G1201_t8	Wound	II	14	> 6*			+
26	121	BU_G2601A_t9	Wound	II	3	3.5			
22	3019	BU_N22_t6	Nose	N/A	N/A	N/A			+
12	508	BU_W12_t13	Wound	II	26	6			
3	152	BU_G0301_t8	Wound	III	15	> 6*	+	+	
10	152	BU_G1074_t4	Wound	III	7	4.5	+	+	
11	152	BU_G1101_t2	Wound	II	3	2.75	+	+	
10	152	BU_G1001_t8	Wound	III	15	4.5	+	+	
17	152	BU_N17W_t2	Nose	N/A	N/A	N/A	+	+	
7	152	BU_G0706B_t8	Wound	II	13	6	+	+	

[a] Abbreviations used: ST, sequence type; N/A, not applicable.
[b] Numbers indicate the category of lesions: I, lesions ≤ 5 cm; II, lesions between 5 and 15 cm; III, lesions ≥ 15 cm or lesions at critical sites such as the eye and genitals.
[c] An * indicates the end of observation.
[d] Hemolytic activity is indicated by a +.

Table 3
Detection of mobile genetic elements and additional virulence genes.

Strains	ST	SaPIa	SaPIb	Prophage φSa3	Prophage φSa2	Prophage φSa6	Genomic island ιSaα	Genomic island ιSaβ
BU_G0201_t8	88	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	set(11), lpl(3)	*lukD, lukE,bsaG, spl*(8)
BU_G0701_t5	88	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	*set*(11), *lpl*(3)	*lukD, lukE, bsaG, spl*(8)
BU_G0202_t2	88	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	*set*(11), *lpl*(3)	*lukD, lukE, bsaG, spl*(8)
BU_G1905_t3	88	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	*set*(11), *lpl*(3)	*lukD, lukE, bsaG, spl*(8)
BU_W13_t1	88	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	*set*(11), *lpl*(3)	*lukD, lukE, bsaG, spl*(8)
BU_W22_t4	5	SaPIGhana1 (*ear)*	–	φSa3 (*sak, chp, scn)*	φSa2 (*sea, lukF, lukS*)	–	*set*(10), *lpl*(10)	*lukD, lukE, seo, sem, sei, yent1, yent2, sen, seg, spl*(3)
BU_W7A_t11	5	SaPIGhana2	–	φSa3 *(sea, sak, chp, scn)*	–	–	*set*(10), *lpl*(9)	*lukD, lukE, seo, sem, sei, yent1, yent2, sen, seg, spl*(5)
BU_N17Y_t2	15	SaPIGhana3 (*ear)*	–	phage remnants *(chp, scn*)	–	φSa6 (*eta*)	*set*(11), *lpl*(8)	*lukD, lukE, spl*(6)
BU_N3_t2	15	–	–	phage remnants *(chp, scn*)	–	–	*set*(11), *lpl*(9)	*lukD, lukE, spl*(4)
BU_W6_t1	1	SaPIGhana4 (*seb*, *sae*, *tst-1)*	SaPIGhana5 (*eta*)	φSa3 *(sea, sak, scn, sel, sek)*	φSa2 (*lukF, lukS*) PVL	–	*set*(11), *lpl*(5)	*lukD, lukE, lukM/lukS, seh, sej, ser, seh, spl*(6)
BU_G1201_t13	121	SaPIGhana6 *(seq*, *sek)*	SaPIGhana7*(seb, ear)*	φSa3 *(sak, scn)*	φSa2 (*lukF, lukS*) PVL	–	*set*(11), *lpl*(3)	*lukD, lukE, sec2, seo, sei, sec3, sen, seg, spl*(3)
BU_G1201_t8	121	SaPIGhana6 *(seq*, *sek)*	SaPIGhana7*(seb, ear)*	φSa3 *(sak, scn)*	φSa2 (*lukF, lukS*) PVL	–	*set*(11), *lpl*(3)	*lukD, lukE, sec2, seo, sei, sec3, sen, seg, spl*(3)
BU_G2601A_t9	121	SaPIGhana6 *(seq*, *sek)*	SaPIGhana7*(seb, ear)*	φSa3 *(sak, scn)*	φSa2 (*lukF, lukS*) PVL	–	*set*(11), *lpl*(3)	*lukD, lukE, sec2, seo, sei, sec3, sen, seg, spl*(3)
BU_N22_t6	3019	–	–	φSa3 (*sak, chp, scn)*	φSa2	–	*set*(9), *lpl*(6)	*seo*, *sek*, *sei*, *sec1*, *sen*, *seg*
BU_W12_t13	508	SaPI68111 (*sel*, *seb*, *ear*, *tst-1)*	–	φSa3 (*sak, chp, scn)*	–	–	*set*(9), *lpl*(6)	*seo, sek, sei, seu, sen, seg, sea*
BU_G0301_t8	152	–	–	φSa3 (*sak, scn)*[a]	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(6)	*lukD, lukE, bsa*(8)
BU_G1074_t4	152	–	–	φSa3 (*sak, scn)*[a]	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(6)	*lukD, lukE, bsa*(8)
BU_G1101_t2	152	–	–	φSa3 (*sak, scn*)a	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(6)	*lukD, lukE, bsa*(8)
BU_G1001_t8	152	–	–	φSa3 (*sak, scn)*[a]	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(6)	*lukD, lukE, bsa*(8)
BU_N17W_t2	152	–	–	φSa3 (*sak, scn)*[a]	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(4)	*lukD, lukE, bsa*(8)
BU_G0706B_t8	152	–	–	φSa3 (*sak, scn)*[b]	φSa2 (*lukF, lukS*) PVL	–	*set*(8), *lpl*(6)	*lukD, lukE, bsa*(8)

[a] Phage φSa3 integrated into *hsdS*.
[b] Phage φSa3 integrated into non-coding region.

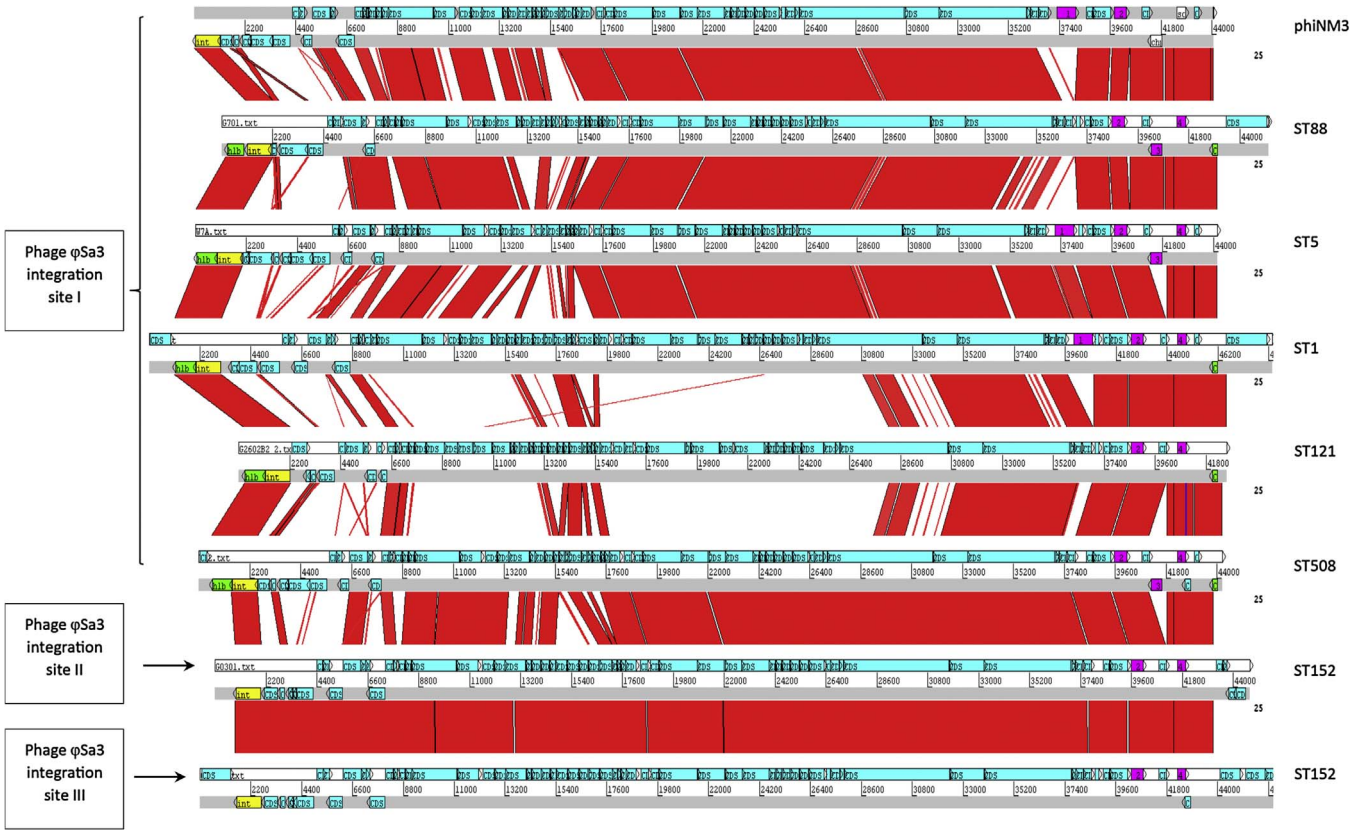

Fig. 1. Diversity of φSa3 phages and their genomic integration sites in sequenced *S. aureus* isolates from BU patients. BLASTN-based alignment (nucleotide identity > 80% shown) of the sequences of φSa3 bacteriophages identified in 21 sequenced Ghanaian *S. aureus* isolates as displayed by the Artemis comparison tool (ACT). The previously published DNA sequence of phage phiNM3 from strain Newman (NCBI number: DQ530361.1) was used as a reference to compare with other bacteriophage sequences included in this alignment. Further, representative φSa3 bacteriophages from *S. aureus* isolates of particular sequence types are shown (ST88 – BU_G0701_t5; ST5 – BU_W7A_t11; ST1 – BU_W6_t1; ST121 – BU_G2601A_t9; ST508 – BU_W12_t13; ST152 – BU_G0301_t8 and BU_G0706B_t8). Red lines between compared sequences highlight orthologous sequences with the same orientation. Identified open reading frames (ORFs) are colored in light blue, the integrase gene is marked in yellow, immune invasion cluster genes are indicated in pink and assigned with specific numbers (1- *sae*; 2 – *sak*; 3 – *chp*; 4 – *scn*). Phage integration points are indicated by roman numbers (I – *hlb* (green); II – *hsdS*; III – non-coding region).

genes were encoded by the identified prophages. The most common prophage type (φSa3) was identified in 19 *S. aureus* isolates. The prophage φSa2 was detected in 17 isolates, but isolates belonging to ST88 and ST3019 contained prophage φSa2 without the genes encoding PVL. One ST15 isolate harbored the φSa6 that encoded the *eta* gene (Table 3).

Interestingly, analysis of the integration sites of φSa3 showed three genomic positions that may be occupied by this phage type (Fig. 1). Most often this phage was found to split the *hlb* gene into two parts as illustrated in Fig. 1 for ST88, ST5, ST1, ST121 and ST508 isolates (top to bottom). This is similar to the situation encountered for phiNM3 in strain Newman (DQ530361.1; Fig. 1) (Bae et al., 2006). Isolates belonging to ST15 carried the IEC gene cluster (*chp* and *scn*) at the *hlb* position, while an intact prophage was missing (not shown). The second locus where the φSa3 prophage had inserted was into the *hsdS* gene, which encodes a specificity subunit of the type I restriction modification system that is present within the β-genomic island (νSaβ). This location of φSa3 was identified in the genomes of five ST152 isolates (Fig. 1 and Table 3). The third genomic position occupied by φSa3 was a non-coding region which, in the reference genome MRSA252, is located between the open reading frames (ORFs) SAR0654 and SAR0655 (Fig. 2A). This location of φSa3 was only found in the genome of one isolate belonging to ST152 (Fig. 1 and Table 3).

Except two ST508 and ST3019 isolates, the genomes of all other isolates carried the *lukD* and *lukE* genes for a pore-forming toxin in the highly variable νSaβ genome region (Table 3).

3.4. Novel pathogenicity islands

Analysis of the sequence data of the 21 isolates revealed the presence of eight different SaPIs. Remarkably, only one had been previously identified whereas the other seven contained novel regions. Furthermore, most of the identified SaPIs had mosaic structures containing regions of known SaPIs as well as completely novel genes not identified previously. We named these new pathogenicity islands SaPIGhana1 to SaPIGhana7 (Table 3). SaPIGhana1 from isolate BU_W22_t4 (ST5) is composed of regions belonging to SaPI2 and harbors the *ear* gene that encodes for a penicillin-binding protein (Fig. 3). Additionally, it contains two novel genes whose functions are yet unknown. SaPIGhana2 from isolate BU_W7A_t11 (ST5) represents a novel SaPI type that is similar to the SaPI from *S. aureus* strain OC3 (ST8) (Accession No. AB983199.1), which was previously isolated in Japan. This SaPI carries the *fhuD* gene, encoding a ferrichrome-binding protein that is important for growth under iron-restricted conditions. Isolate BU_N17Y_t2 (ST15) contains SaPIGhana3, which is a SaPI1-like element with a novel region with three genes of unknown function downstream of the terminase gene. The isolate BU_W6_t1 (ST1) contains two SaPIs. First, we found SaPIGhana4 that shares homologous regions with SaPI1 and contains a novel region that includes the *tst* and *ear* genes plus a truncated *seb gene*. The second pathogenicity island found in this isolate, SaPIGhana5, shares homologous regions with SaPImw2. However, this SaPI contains a novel region downstream of the terminase gene with three novel genes and the *eta* gene. Three of the isolates belonging to ST121 also contained two novel SaPIs. SaPIGhana6 (Table 3) is composed of regions similar to two different

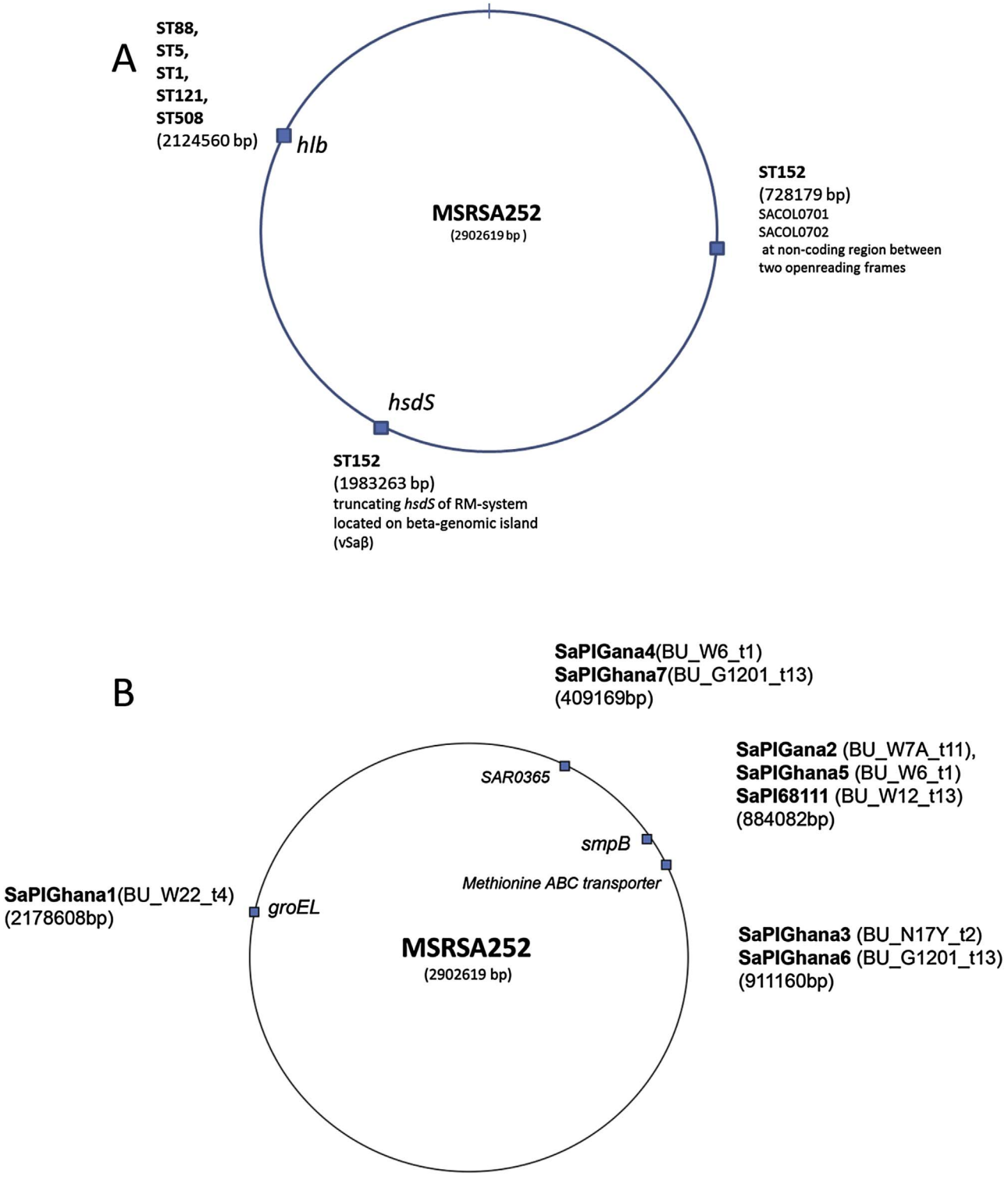

Fig. 2. Genomic positions of integrated φSa3 phages (A) and SaPIs (B). The identified φSa3 phages and SaPIs in sequenced *S. aureus* isolates are projected onto the MRSA252 reference genome. The integration sites are indicated with blue squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pathogenicity islands namely SaPImw2 and SaPIj11, and it harbors the two enterotoxin genes *sek* and *seq*. The second SaPI, SaPIGhana7, harbors both novel regions and contains regions with high similarity to the previously identified SaPIIVm10 (SaPI4). Further, SaPIGhana7 carries the *seb* and *ear* genes. Lastly, the SaPI identified in isolate BU_W12_t13 (ST508) shares overall similarity with the previously identified SaPI68111. This SaPI carries the *tst*, *ear*, *seb* and *sel* genes. Amongst the analyzed isolates, SaPIs were identified at four different genomic positions as mapped on the reference genome of the MRSA252 strain (Fig. 2B). SaPIGhana4 and 7 had integrated upstream the gene coding for the hypothetical protein SAR0365. A second SaPI integration point, where SaPIGhana2, 5 and SaPI68111 were identified, was located downstream of the *smpB* gene coding for the putative tmRNA-binding protein SAR0837. A third integration point, where SaPIGhana 3 and 6 were found, was located downstream of the ORF coding for the methionine ABC transporter SAR0871. The fourth integration site

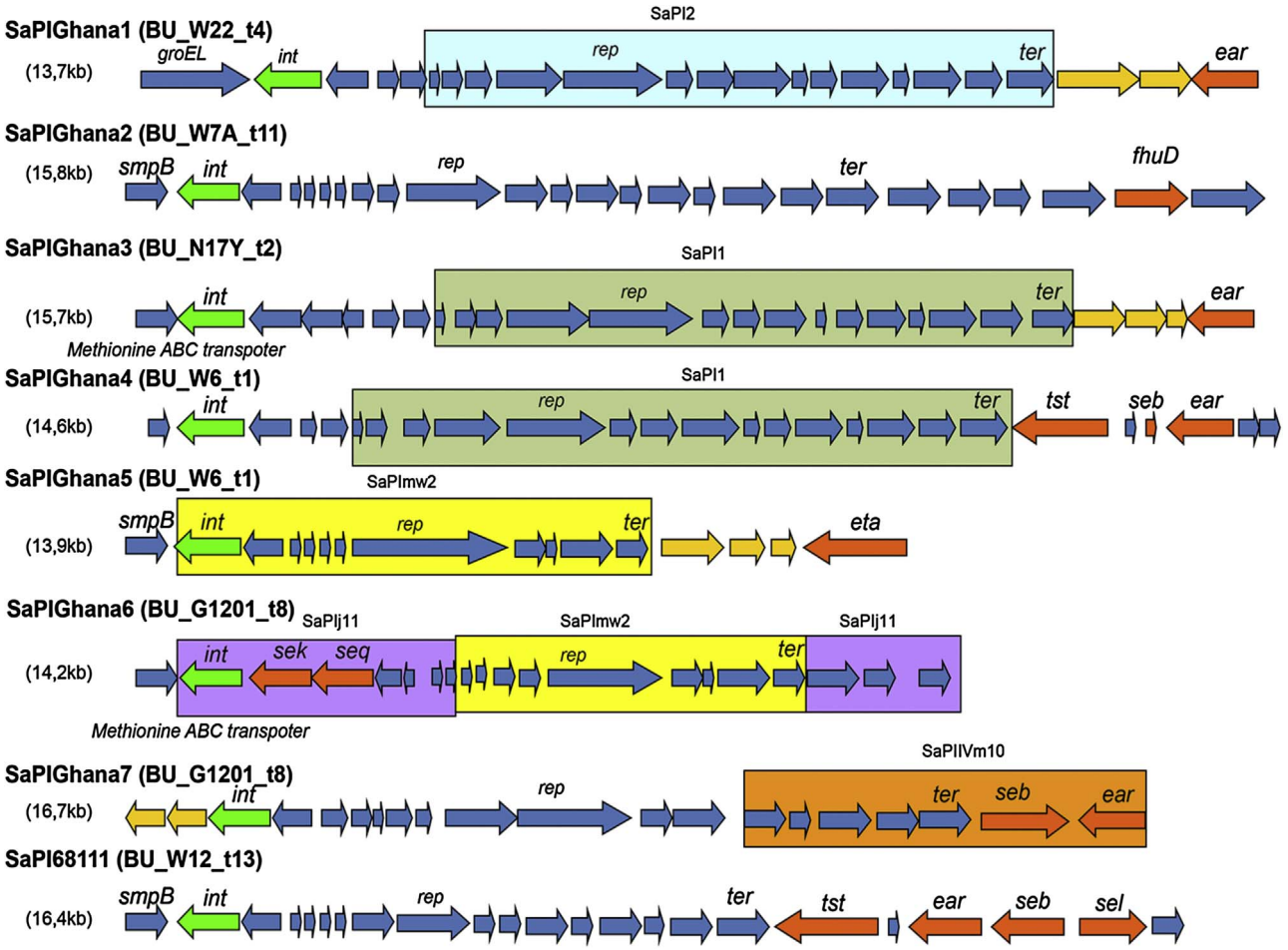

Fig. 3. Diversity of SaPIs and their integration sites in sequenced *S. aureus* isolates from BU patients. Open reading frames (ORFs) are indicated by arrows. Homologous regions to known SaPIs are indicated by colored blocks. Hypothetical ORFs are indicated in blue; the SaPI integrase gene (*int*) is marked in green; genes encoding virulence factors are indicated in orange; novel ORFs are indicated by yellow arrows. *ter* represents the SaPI terminase gene.

containing SaPIGhana1 was located downstream of the *groEL* gene.

3.5. Genomic regions downstream the SCC*mec* insertion site in *S. aureus* isolates from BU patients

The integration site of known SCC*mec* elements is localized at the *rlmH* gene (previously designated *orfX*), encoding a methyltransferase. A detailed inspection of the region downstream of *rlmH* in the sequenced MRSA and MSSA isolates showed that it was conserved in isolates of the same sequence type. However, it differed substantially with respect to gene content in isolates of different sequence types. Novel genomic regions downstream of *rlmH* integration sites were found in sequenced isolates belonging to ST88, ST152 and ST3019. Five of the investigated ST88 isolates were MRSA that carried SCC*mec* IVa. Interestingly, all these five isolates contained a novel DNA fragment of 7 kb downstream of the SCC*mec* element that includes eight open reading frames of unknown function (Fig. 4). In this fragment, a stretch of 3.6 kb shares 89% sequence similarity with a corresponding region of the SCC element present in *S. haemolyticus* strain SH480 (GenBank: AB477967.1).

Of note, a novel region of 5 kb in size was identified downstream of *rlmH* in ST152 MSSA isolates. This region contains six novel genes encoding hypothetical proteins (Fig. 4). The one investigated ST3019 MSSA isolate carried an SCC element with a *cap* operon that encodes for capsular polysaccharide in *S. aureus*. Downstream of this novel SCC, a new genomic region of 10 kb in size was identified that contained 9 novel open reading frames not reported before. Further, five different but previously characterized genomic regions downstream of *rlmH* were encountered in the remaining MSSA isolates. Specifically, in the ST5 MSSA isolates the analyzed genomic region downstream of *rlmH* was identical to that of the *S. aureus* isolates ED98 (GenBank: CP001781.1) and 502A (GenBank: CP007454.1). In ST15 isolates the region downstream of *rlmH* was identical to the respective region of the previously reported 15666 SCC*mec* insertion site (Noto et al., 2008). The ST1 MSSA isolate had a genomic region downstream of *rlmH* identical to the region described previously as the 15575 SCC*mec* insertion site genomic sequence, which contains the gene for the enterotoxin Seh. The MSSA isolates with ST121 contained genomic regions downstream of *rlmH* that were identical to the respective region described as the 3289 SCC*mec* insertion site genomic sequence, which carries the *sec2* enterotoxin gene. The genomic region downstream *rlmH* in the ST508 isolate is identical to the corresponding region of MRSA strain CA-347 (GenBank: CP006044.1) (Fig. 4). This region was found to encode a restriction-modification system (*hsdR hsdM*) and to include a MGE encoding an ErmB/QacA drug resistance transporter of the major facilitator superfamily (MFS).

Lastly, all investigated isolates contained genomic islands νSaα and νSaβ with varying sets of genes encoding exotoxins, enterotoxins, lipoproteins and serine proteases that target elements of the innate immune response (Table 3). In addition, the ST1 isolate was found to encode a third pore-forming leukocyte toxin, encoded by the *lukM* and *lukS* genes.

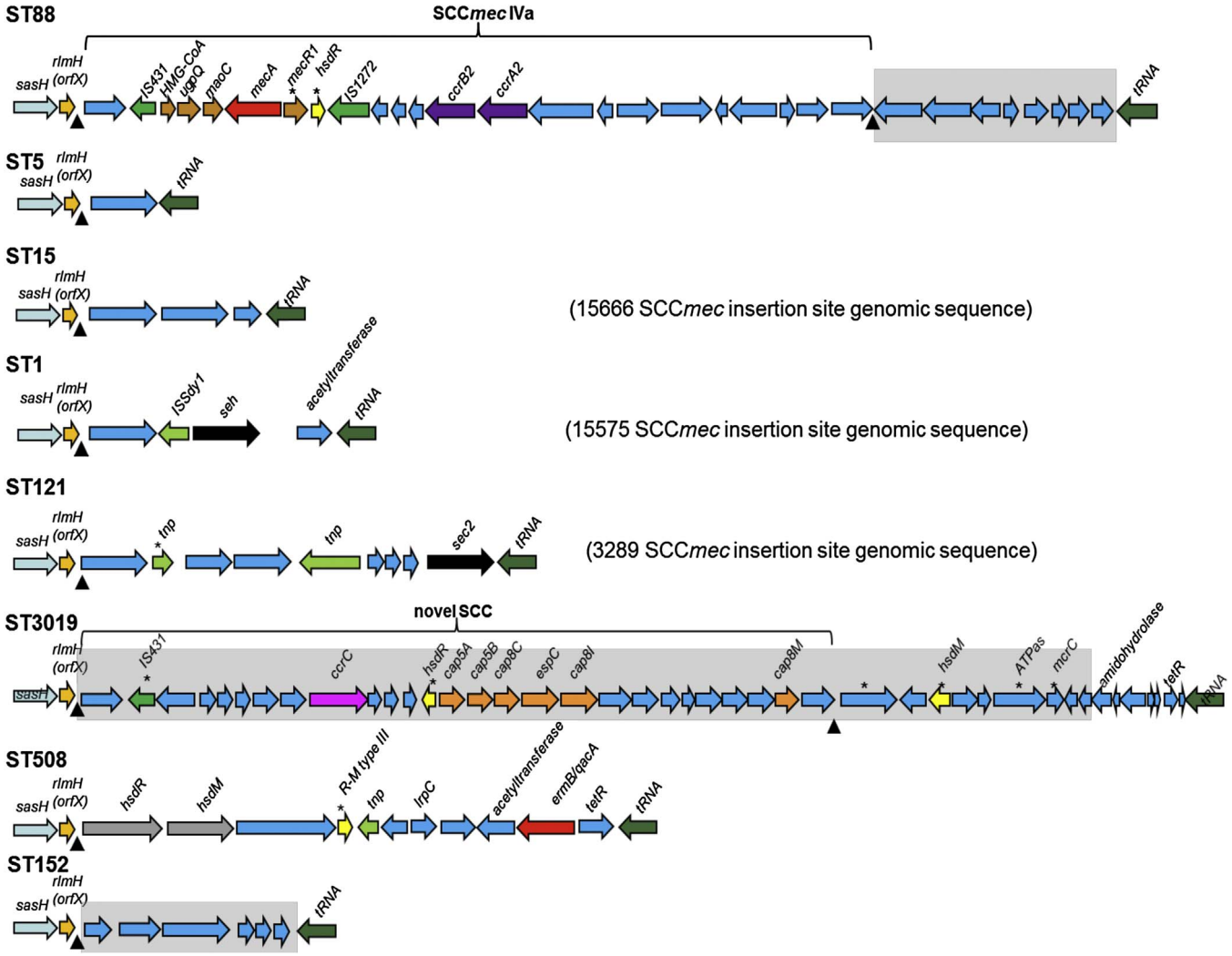

Fig. 4. Structure of genomic regions downstream of SCC*mec* insertion sites in sequenced *S. aureus* isolates from BU patients. Open reading frames (ORFs) are marked by arrows and truncated ORFs are indicated by * above the arrow; *rlmH* (*orfX*) is indicated in yellow; hypothetical ORFs are indicated in blue; genes coding for antibiotic resistance are indicated in red; genes encoding virulence factors are indicated in black; and direct repeats are marked by a black arrow head. The novel genomic regions present in isolates of ST88, ST152 and ST3019 are indicated with grey shading.

4. Discussion

The pathogenesis of BU is associated with mycolactone, the main virulence factor of *M. ulcerans* that is responsible for tissue necrosis and suppression of the immune response of BU patients (George et al., 2000, 1999). The resulting wounds are subsequently colonized with different bacteria, including *S. aureus* (Amissah et al., 2015b; Barogui et al., 2013). We hypothesize that *S. aureus* is one of the drivers of delayed wound healing, besides other factors such as poor nutrition and inadequate wound management. In the ideal situation, wound management in BU should include rinsing with saline, and the application of vaseline gauze topped with absorptive dressing material and compression, preferably with a short-stretched bandage. Poor wound management and transmission events during wound care predispose the wounds of BU patients to colonization with microorganisms including *S. aureus* (Amissah et al., 2015a; Velding et al., 2014). Hence small lesions (< 10 cm) that are expected to heal within two to four weeks of antibiotic therapy take longer time to heal. For example, in one large trial with drug treatment alone, it took patients a median 18 weeks to heal (Nienhuis et al., 2010). This may at least partially be due to the colonization with bacteria including *S. aureus*. In our present study, it took 6 months for most category II wounds colonized with *S. aureus* to heal (Table 2) compared to 3–4 months for patients not colonized with *S. aureus* (data not shown). Furthermore, temporal changes in *S. aureus* genotypes were observed in the wounds of some BU patients (Amissah et al., 2015b). Here, we observed different *S. aureus* genotypes containing additional virulence genes over time (patients 7 and 12) (Table 3). Thus BU wounds that are not healed after two months of antibiotic therapy may even be re-colonised with new *S. aureus* genotypes that can be more virulent than the original colonizing *S. aureus*. In this respect, it is noteworthy that the *S. aureus* genotypes identified in BU wounds have also been detected in the nares, skin and soft tissue infections, and bacteraemia in Ghana and other African countries (Conceicao et al., 2015; Egyir et al., 2014; Kraef et al., 2015; Ruimy et al., 2008; Shittu et al., 2012). In fact, in Ghana, *S. aureus* contributes largely to bacteraemia (10.8–13.2%) (Anyebuno and Newman, 1995; Nielsen et al., 2012). Further, patients with skin infections such as BU are heavily colonized with *S. aureus* (Amissah et al., 2015b; Barogui et al., 2013). The BU lesions are often colonized by *S. aureus* during disease management, i.e. during and after antimicrobial therapy and wound management (Amissah et al., 2015b). In this respect, it should be noted that none of the patients in this study were prescribed antibiotics other than the topical antibiotics used during wound dressing.

The present study characterized the virulence genes in *S. aureus* isolated from BU patients. Genes for staphylococcal virulence factors are often encoded by MGEs, such as prophages, plasmids, genomic islands and SaPIs. Accordingly, the present study attributed particular

attention to MGEs and the encoded virulence and resistance genes. In many isolates α-, β- and δ-hemolysin genes were found, whereas their activity was only detected in some investigated *S. aureus* isolates. In the case of two *agr*-positive MSSA isolates (ST121 and ST508; *agr* type IV; Table 1) no hemolytic activity was detectable. For the respective ST508 isolate, this phenotype may be explained by multiple mutations in the *agrC* locus and RNAIII. On the other hand, in the non-hemolytic ST121 isolate, the only mutation detected in the *agr* locus concerned the insertion of one adenine in the RNAIII region. It is presently not clear whether this explains the non-hemolytic phenotype. Alternatively, it is conceivable that the lack of hemolytic activity was due to a suppression of *agr* function by upstream regulators, such as *sigB,* as was previously reported for glycopeptide-intermediate resistant *S. aureus* (Bischoff et al., 2001; Bischoff and Berger-Bachi, 2001; Renzoni et al., 2004; Sakoulas et al., 2006). The latter observation has been associated with the ability of MRSA to survive under glycopeptide selective pressure. Further studies are needed to understand the mechanism of the loss of hemolytic activity in the MSSA isolates under rifampicin selection pressure against which one isolate is resistant. Of note, the method used in the detection of hemolytic activity may not be sensitive enough for the detection of weak α-hemolysin activity, which is a potential limitation in this study. Conceivably, the pore-forming activity of the detected hemolysins may contribute to tissue necrosis in wounds of BU patients after antibiotic therapy of the primary infection caused by *M. ulcerans*. On the other hand, the virulence of some isolates in our study may be affected to some extent by the integration of a prophage or the IEC gene cluster in the *hlb* gene. Indeed such genetic changes have been reported to have the potential to enhance the pathogenicity of the isolates (Salgado-Pabón et al., 2014). Intriguingly, four isolates belonging to ST88 showed high a β-hemolysin-like activity despite the insertion of a prophage in *hlb*. This implies that an as yet unidentified other factor with β-hemolysin-like activity is responsible for this phenotype.

MGEs such as (pro)phages represent a driving force in staphylococcal host adaptation and infection (Wagner and Waldor, 2002). Consistent with this view, almost all isolates carried phage-encoded virulence factors, particularly those involved in phagocyte evasion by inhibiting phagocytosis (CHIPS, SCIN, Sak, and Sea) and by directly attacking phagocytes (PVL) (van der Vijver et al., 1972). The insertion of the φSa3 prophage, which encodes CHIPS, SCIN, Sak and Sea, into the *hlb* gene has been reported to serve as a regulator of virulence gene expression by increasing fitness and virulence in new infection niches (Salgado-Pabón et al., 2014). It seems likely that this will also be true for most of the presently investigated isolates.

Seven novel SaPIs were identified in the sequenced *S. aureus* isolates from BU patients. These were shown to carry genes for virulence factors, such as *ear*, *seb*, *sae*, *sek*, *sel*, *seq*, *tst*-1 and *eta*, suggesting that the respective staphylococci have the ability to cause infections like sepsis, toxic shock syndrome and scalded skin syndrome (Fraser et al., 2000; Holtfreter and Bröker, 2005; Yamasaki et al., 2005). More importantly, certain combinations of staphylococcal toxin genes reported to be rare and associated with mortality were found in the investigated isolates (Ambrozova et al., 2013). For instance, SaPIGhana4 encoding *tst*-1, *seb*, *sae*, φSa2 encoding *lukF* and *lukS*, φSa3 encoding *sea, sak, chp, scn, sel, sek*, and the additional virulence factors *lukM/lukS, seh, sej, ser, seh* were detected in the genomes of *S. aureus* isolated from wounds. Further, the enterotoxin-encoding genes *seg*, *sei*, *sem*, *sen*, *seo* located in the enterotoxin gene cluster (*egc*) were often identified in isolates harboring the novel SaPIs. Epidemiological data suggests that *egc* facilitates the colonization of mucosal surfaces, which precedes local and invasive infection and is associated with lower disease-invoking potential as compared to *S. aureus* superantigens (Ferry et al., 2005). This may be one reason why serious infections, such as sepsis or toxic shock syndrome, did not occur in the BU patients from whom the investigated *S. aureus* isolates were obtained. Indeed, none of the patients were clinically suspected of having a secondary infection with or were treated for a suspected *S. aureus* infection. This suggests that the presence of certain virulence genes may counteract the effects of other virulence factors. It also suggests that multiple virulence factors encoded on MGEs may contribute differentially to the survival and persistence of *S. aureus* in the wound. However, the confirmation of this idea awaits experimental verification through expression analyses and testing the isolates in appropriate animal infection models.

The known SCC*mec* integration site located at *rlmH* (*orfX*) deserves special attention also in MSSA isolates, because this genomic region seems to be attractive for integration of other non-SCC*mec* elements that may encode virulence or fitness-enhancing determinants. This was clearly the case in isolates belonging to ST1, ST121 and ST508 that were found to harbor at this locus the enterotoxins genes *seh* or *sec2*, or the gene encoding an EmrB/QacA drug resistance transporter, respectively (Amissah et al., 2015b). The identification of an SCC element with a *cap* operon in an *S. aureus* ST3019 isolate suggests that this isolate may have acquired a fitness advantage as well as a phagocytosis-resistance phenotype (Luong and Lee, 2002; Pardo et al., 2009; Voyich et al., 2005). However this needs to be further investigated.

The virulence genes *sea, sei*, *lukDE*, *lukF*-PV, *lukS*-PV, *hlgv* and *cap*8 have been associated with moderate to severe infections in diabetic foot ulcers (grades 2–4) (Messad et al., 2013; Sotto et al., 2008). For *S. aureus* isolates that cause invasive infections, the *cap*5, *lukDE* and *scn* genes, as well as genes encoding fibronectin-binding protein (*fnbB*), serine proteases A and B (*splA/splB*), and staphylococcal exotoxin-like proteins (*setC* or *seIX*) have been reported to be frequently present (Rasmussen et al., 2013). Although some virulence genes are reported to be associated with severe infections in diabetic ulcers and in patients with invasive staphylococcal disease, this study lacks the metadata to make such inference. Nonetheless, one observation is noteworthy in this respect, namely that the nasal isolates from patients 3 and 22 seem to have fewer SaPIs, prophages and virulence genes than the wound isolates from these patients (Table 3). However, it should be noted that the respective nasal and wound isolates belonged to different sequence types, which makes it difficult to speculate about possible differences in the virulence of these isolates.

In conclusion, the sequence data of *S. aureus* isolates from BU patients uncovered a large but variable reservoir of MGEs and virulence genes that may enhance their survival and persistence in the human host. In particular, we identified seven novel enterotoxin-encoding SaPIs from BU patients. It should however, be noticed that this study provides only a first inventory of the virulence potential of the investigated isolates. Accordingly, we cannot yet correlate clinical data (*e.g.* percentage change in wound size over time) with the presence or absence of particular virulence genes. In addition, the virulence potential or presence of other bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii, Klebsiella pneumonia* and *S. haemolyticus,* isolated from BU wounds could contribute to the delay in wound healing of patients as well (Amissah et al., 2015b). Consequently, without further studies on the expression of virulence factors of wound-resident *S. aureus* and other microorganisms, the role of *S. aureus* in potentially causing delay in wound healing is presumptive and needs to be proven in further research.

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Author contributions statement

N.A.A, M.A.C, A.A, T.S.vdW, J.M.vD, J.W.R and Y.S conceived and designed the experiments, N.A.A, M.A.C, C.S.T and I.P conducted the experiments, N.A.A, M.A.C, J.M.vD and J.W.R analysed the results, M.A.C, J.M.vD, A.A, A.W.F, T.S.vdW, J.W.R and YS contributed reagents/materials/analysis tools. All authors reviewed and approved the final manuscript.

Additional information

Accession codes: LGAE00000000, LFTW00000000, LFTV00000000, LFTU00000000, LFTT00000000, LFOH00000000, LFOG00000000, LFNS00000000, LFNR00000000, LFNQ00000000, LFNP00000000, LFNO00000000, LFNN00000000, LFNM00000000, LFNL00000000, LFNK00000000, LFNJ00000000, LFNI00000000, LFNH00000000, LFMH00000000, LFMG00000000.

Competing financial interests

The authors declare no competing financial interests.

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